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Species-Level Identification of Isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* Complex by Sequence Analysis of the 16S-23S rRNA Gene Spacer Region

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The species *Acinetobacter calcoaceticus*, *A. baumannii*, genomic species 3, and genomic species 13TU included in the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex are genetically highly related and difficult to distinguish phenotypically. Except for *A. calcoaceticus*, they are all important nosocomial species. In the present study, the usefulness of the 16S-23S rRNA gene intergenic spacer (ITS) sequence for the differentiation of (genomic) species in the *A. calcoaceticus-A. baumannii* complex was evaluated. The ITSs of 11 reference strains of the complex and 17 strains of other (genomic) species of *Acinetobacter* were sequenced. The ITS lengths (607 to 638 bp) and sequences were highly conserved for strains within the *A. calcoaceticus-A. baumannii* complex. Intraspecies ITS sequence similarities ranged from 0.99 to 1.0, whereas interspecies similarities varied from 0.86 to 0.92. By using these criteria, 79 clinical isolates identified as *A. calcoaceticus* (18 isolates) or *A. baumannii* (61 isolates) with the API 20 NE system (bioMérieux Vitek, Marcy l'Etoile, France) were identified as *A. baumannii* (46 isolates), genomic species 3 (19 isolates), and genomic species 13TU (11 isolates) by ITS sequencing. An identification rate of 96.2% (76 of 79 isolates) was obtained by using ITS sequence analysis for identification of isolates in the *A. calcoaceticus-A. baumannii* complex, and the accuracy of the method was confirmed for a subset of strains by amplified rRNA gene restriction analysis and genomic DNA analysis by AFLP analysis by using libraries of profiles of reference strains. In conclusion, ITS sequence-based identification is reliable and provides a promising tool for elucidation of the clinical significance of the different species of the *A. calcoaceticus-A. baumannii* complex.

Since the 1970s, bacteria of the genus *Acinetobacter* have increasingly been recognized as important nosocomial pathogens that give rise to severe infections and episodes of epidemic spread among critically ill hospitalized patients (3, 13, 31, 32). In a recent European study, *Acinetobacter* spp. appeared to be the eighth most common cause of nosocomial pneumonia (17). Nosocomial acinetobacters are notorious for their resistance to antibiotics, and strains resistant to most or all clinically important antibiotics, including expended-spectrum β-lactams and carbapenems, have now been identified worldwide (1, 5, 11, 26, 41).

The genus *Acinetobacter* currently contains up to 33 described named and unnamed (genomic) species (9, 35). Of these, *Acinetobacter calcoaceticus*, *A. baumannii*, and genomic species 3 and 13TU are genetically and phenotypically very similar (21), which has led to the proposal to lump these and two closely related genomic species (genomic species close to 13TU and genomic species between 1 and 3) into the *A. calcoaceticus-A. baumannii* complex (20). The lumping of these species is unsatisfactory for clinical reasons because it obscures possible differences in the biology and pathology of the individual species. For example, *A. calcoaceticus* is considered a soil organism (6), while the other three species are clinically the most important *Acinetobacter* species, with *A. baumannii* apparently being mostly involved in infection and epidemic spread. In addition, Houang et al. (25) reported significant differences in antimicrobial susceptibilities among isolates of *A. baumannii*, genomic species 3, and genomic species 13TU. Therefore, precise identification of the species in the *A. calcoaceticus-A. baumannii* complex is important to elucidate the ecology, epidemiology, and pathology of these species; and practical identification methods must be developed for this purpose (25, 41).

Phenotypic identification of *Acinetobacter* isolates to the species level has proven to be insufficient (15, 21). However, several genotypic methods have been developed for (genomic) species identification of acinetobacters, including the *A. calcoaceticus-A. baumannii* complex. These methods include restriction analysis of amplified ribosomal DNA (ARDRA) (14, 44), the 16S-23S rRNA gene intergenic spacer (ITS) region (16), the whole ribosomal operon (18), and the *recA* gene (39).

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TABLE 1. Type and reference strains used in this study and intraspecies similarities of the 16S-23S rRNA gene ITS regions of species in the *A. calcoaceticus-A. baumannii* complex

Species	Strain[a]	Other strain designation	ITS length (bp)	Similarity[b]	GenBank accession no.	Reference(s)
A. calcoaceticus	LMG 1046^T		638		AY601820	6
	LMG 992		638	1.00	AY601821	6
	BCRC 11562	ATCC 14987	637	0.99	AY601822	6
A. baumannii	BCRC 10591^T	ATCC 19606^T	607		AY601823	6
	BCRC 15884	ATCC 15151	607	1.00	AY601824	6
	BCRC 15886	ATCC 19003	607	1.00	AY601825	6
	LMG 984		607	1.00	AY601826	6
Genomic species 3	LMG 1035		619		AY601827	6
	CCUG 26384		619	1.00	AY601828	43
	BCRC 15420	ATCC 17922	619	1.00	AY601829	6
Genomic species 13TU	BCRC 15417	LUG 993	615		AY601830	6
A. haemolyticus	BCRC 14852^T	ATCC 17906^T	614[c]		AY601831	6
A. junii	BCRC 14854^T	ATCC 17908^T	706		AY601832	6
Genomic species 6	BCRC 15421	ATCC 17979	636		AY601833	6
A. johnsonii	BCRC 14853^T	ATCC 17909^T	703[c]		AY601834	6
A. lwoffii	BCRC 14855^T	ATCC 15309^T	629[c]		AY601835	6
Genomic species 10	BCRC 15423	ATCC 17942	613[c]		AY601837	6, 47
Genomic species 11	BCRC 15424	ATCC 11171	593[c]		AY601838	6
A. radioresistens	BCRC 15425^T	ATCC 43998^T	632		AY601839	37
Genomic species 14TU (13BJ)	LMG 1235	ATCC 17905	667[c]		AY601840	47
Genomic species 15TU	CCUG 26390		661		AY601841	43
Genomic species 14BJ	CCUG 34435		620[c]		AY601842	8
Genomic species 15BJ	CCUG 34436		667		AY601843	8
Genomic species 16	BCRC 15883	ATCC 17988	595		AY601844	8
Genomic species 17	CCUG 34437		655		AY601845	8
"A. venetianus"	CCUG 45561^T		612		AY601846	45
A. ursingii	LMG 19575^T		715[c]		AY601847	34
A. schindleri	LMG 19576^T		638		AY601848	34

[a] ATCC, American Type Culture Collection, Manassas, Va.; BCRC, Bioresources Collection and Research Center, Hsichu, Taiwan; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; LMG, Laboratorium voor Microbiologie, Ghent, Belgium.
[b] The type strain or the first strain of each genomic species was used as the basis for the calculation of similarity.
[c] Strain containing multiple ITS sequences; only the shortest one is described here.

Ribotyping (19) and analysis of DNA fingerprints generated by selective amplification of whole-genome restriction fragments by AFLP (Keygene, Wageningen, The Netherlands) (30, 34) have also been used successfully.

With the increasing availability of sequencing facilities, sequences of specific genes may be useful for species identification. Partial (42) or nearly complete (27) sequence analyses of the 16S rRNA gene for *Acinetobacter* classification have been reported. In addition, the ITS region separating the 16S and 23S rRNA genes has been suggested to be a good candidate for bacterial species identification (2), since these regions have degrees of low intraspecies variation and high degrees of interspecies divergence (10, 46). The aim of this study was to investigate the feasibility of using the ITS sequence to identify members of the *A. calcoaceticus-A. baumannii* complex to the (genomic) species level. For this purpose, a set of reference strains of 21 described (genomic) species was used to determine the intra- and interspecies similarities of the ITS sequences. Next, the intra- and interspecies criteria obtained were used to identify the species of a set of clinical isolates. For a subset of clinical strains, the accuracy of ITS sequencing for species identification of the *A. calcoaceticus-A. baumannii* complex was verified by both ARDRA and AFLP, which have both been validated by using a large set of reference strains (14, 34, 44).

MATERIALS AND METHODS

Bacterial strains. A total of 28 type and reference strains, including 11 strains of four (genomic) species of the *A. calcoaceticus-A. baumannii* complex and 17 strains of 17 other named and unnamed *Acinetobacter* species, were obtained from public culture collections (Table 1). All strains were identified by DNA-DNA hybridization in previous taxonomic studies. In addition, 79 clinical isolates phenotypically identified as *A. calcoaceticus* (18 strains) or *A. baumannii* (61 strains) with the API 20 NE system (bioMérieux Vitek, Marcy l'Etoile, France) were obtained from National Cheng Kung University Medical Center (Tainan, Taiwan). Most of these strains were isolated from blood or sputum. All strains were subcultured on tryptic soy agar, incubated at 35°C for 18 to 20 h, and then used for DNA extraction.

DNA preparation. The boiling method described by Vaneechoutte et al. (44) was used to extract the DNA from the bacteria. Briefly, one colony of a pure culture was suspended in 50 μl of sterilized water and heated at 100°C for 15 min. After centrifugation in a microcentrifuge (6,000 × *g* for 3 min), the supernatant was stored at −20°C for further use.

Amplification of ITS region and nucleotide sequence determination. The bacterium-specific universal primers 1512F (5′GTCGTAACAAGGTAGCCGTA 3′) and 6R (5′GGGTTYCCCCRTTCRGAAAT3′) (where Y is C or T and R is A or G) (40) were used to amplify a DNA fragment that encompassed a small fragment of the 16S rRNA gene region, the ITS, and a small fragment of the 23S rRNA gene region. The 5′ end of primer 1512F is located at position 1493 of the 16S rRNA gene, and the 5′ end of primer 6R is located at position 108 downstream of the 5′ end of the 23S rRNA gene (*Escherichia coli* numbering). PCR was performed with 5 μl (1 to 5 ng) of template DNA in a total reaction volume of 50 μl consisting of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxyribonucleoside triphosphates (0.2 mM each), 1 μM (each) primer, 1 U of *Taq* DNA polymerase, and 50 μl of a mineral oil overlay. The PCR program consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (62°C for 1 min), and extension (72°C

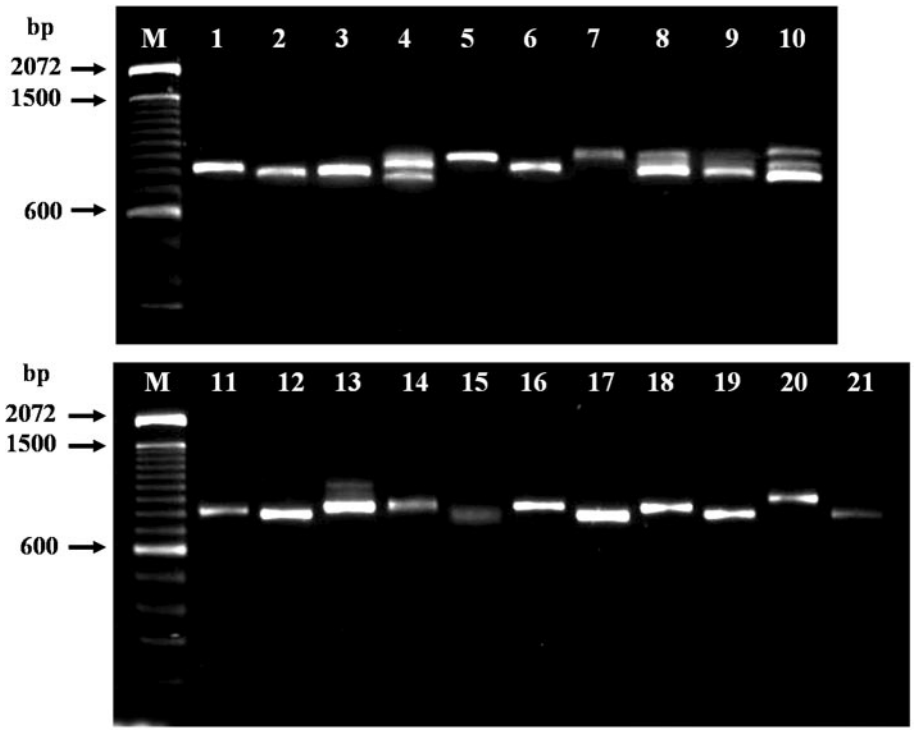

FIG. 1. Amplification of *Acinetobacter* spp. with primers 1512F and 6R and separation of the PCR products by 2% agarose gel electrophoresis. Lanes: M, 100-bp DNA ladder; 1, *A. calcoaceticus*; 2, *A. baumannii*; 3, genomic species 3; 4, *A. haemolyticus*; 5, *A. junii*; 6, genomic species 6; 7, *A. johnsonii*; 8, *A. lwoffii*; 9, genomic species 10; 10, genomic species 11; 11, *A. radioresistens*; 12, genomic species 13TU; 13, genomic species 14TU; 14, genomic species 15TU; 15, genomic species 14BJ; 16, genomic species 15BJ; 17, genomic species 16; 18, genomic species 17; 19, *A. venetianus*; 20, *A. ursingii*; 21, *A. schindleri*.

for 1 min), with a final extension step at 72°C for 7 min. An OmniGen thermal cycler (Hybaid Limited, Middlesex, United Kingdom) was used for PCR.

PCR products were purified with a PCR-M Clean Up kit (Viogene, Taipei, Taiwan) and were sequenced on a model 377 sequencing system (Applied Biosystems, Taipei, Taiwan) with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems). During analysis of the sequences obtained, the portions of the 16S and 23S rRNA gene regions were removed from the sequence data to obtain the exact ITS sequences. For all 28 reference strains of *Acinetobacter* analyzed, the 5′ end of the ITS sequences was ACGAAAGATT, whereas the 3′ end sequence was GGGGTTGTAT (the GenBank accession numbers are given in Table 1). The similarity of the ITS sequence of a strain was obtained by comparing its sequence with that of the type strain or the first reference strain of the same species listed in Table 1, and the PileUp command of the Wisconsin Genetics Computer Group package (version 10.3; Accelrys Inc., San Diego, Calif.) was used to obtain the ITS similarity scores (10).

The PCR products of some named and unnamed species producing more than one amplicon were cloned with a Topo TA cloning kit (Invitrogen Corp., Carlsbad, Ca.), and the ITS fragments of positive clones were reamplified from these clones and sequenced in accordance with the instructions of the manufacturer.

Identification of clinical isolates of *A. calcoaceticus-A. baumannii* complex by ITS sequencing. To evaluate the feasibility of using the ITS sequence for the identification of (genomic) species in the *A. calcoaceticus-A. baumannii* complex, a total of 79 clinical isolates were tested. These isolates were first identified as either *A. calcoaceticus* (18 isolates) or *A. baumannii* (61 isolates) with the API 20 NE system (bioMérieux Vitek). The ITS sequence of a clinical isolate was compared to those of the type strains or the reference strains listed in Table 1, and a species designation was made from the highest similarity score obtained. Two strains of each (genomic) species whose identities by ITS sequencing and with the API 20 NE system were discrepant, including three strains that could not be identified by ITS analysis, were further investigated by both ARDRA and AFLP.

Identification of clinical isolates by ARDRA. The ARDRA method was carried out as described previously (14, 36). Briefly, the amplified 16S rRNA gene was obtained by PCR, and then separate aliquots were digested with five restriction endonucleases (CfoI, AluI, MboI, RsaI, and MspI). The fragments obtained by digestion with each enzyme were electrophoretically separated in 2.5% agarose gels. Species identification was done by comparing the profiles consisting of the combination of restriction patterns generated with the different enzymes to those of a library of profiles of strains of described named and unnamed species (14; http://allserv.rug.ac.be/~mvaneech/ARDRA/Acinetobacter.html).

Identification of clinical isolates by AFLP. The AFLP method was performed as described by Nemec et al. (34). Briefly, EcoRI and MseI were used for restriction, and a primer consisting of MseI with or without a C residue and a primer consisting of Cy5-labeled EcoRI with an A residue (where A and C are selective nucleotides) were used for amplification. The ALFexpress DNA analysis system (Amersham Biosciences, Roosendaal, The Netherlands) was used for fragment separation. Fragments of 50 to 500 bp were used for cluster analysis of strains by using the BioNumerics (version 2.0) software package (Applied Maths, Sint-Martens-Latem, Belgium), the Pearson product moment coefficient (r) as the similarity measure, and the unweighted pair group average linked method for grouping. For species identification, the profile of an isolate was grouped with those in the library of the Leiden University Medical Centre, which contains the profiles of >200 reference strains of all described (genomic) species. An isolate was identified as the species with which it grouped at a level of 50% or greater (34).

Nucleotide sequence accession numbers. The ITS sequences of 11 reference strains of the *A. calcoaceticus-A. baumannii* complex and 17 strains of other *Acinetobacter* (genomic) species were submitted to GenBank. The accession numbers are listed in Table 1.

RESULTS

Amplification and sequencing of ITS fragments. The ITS fragments of 28 reference strains of 21 *Acinetobacter* (genomic) species were amplified by PCR with primers 1512F and 6R. A single amplicon was observed for most (genomic) species, in-

cluding the *A. calcoaceticus-A. baumannii* complex (Fig. 1). However, multiple PCR products were produced by strains belonging to the species *A. haemolyticus*, *A. johnsonii*, and *A. lwoffii* and the unnamed species 10, 11, 14TU, and 14BJ (Fig. 1). For strains with multiple ITS fragments, the ITSs were cloned and then sequenced after reamplification. It was noted that although some strains produced a single band on agarose gel electrophoresis (e.g., Fig. 1, lane 7), actually, more than one amplicon with small differences in lengths was produced by PCR. For example, *A. johnsonii* BCRC 14853 (the type strain of the species) produced two ITS fragments of 703 and 716 bp, respectively (Fig. 1, lane 7).

Among members of the *A. calcoaceticus-A. baumannii* complex, *A. baumannii* had the shortest ITS fragment (607 bp), whereas *A. calcoaceticus* yielded the longest ITS fragment (637 to 638 bp) (Table 1). The intraspecies variation in the lengths of the ITS regions of reference strains of *A. calcoaceticus*, *A. baumannii*, and genomic species 3 was within 1 bp (Table 1).

Sequence similarities of ITS regions. The intraspecies similarities of members of the *A. calcoaceticus-A. baumannii* complex are shown in Table 1. The intraspecies ITS similarities of strains within the *A. calcoaceticus-A. baumannii* complex were very high, ranging from 0.99 to 1.0. However, the intraspecies similarity was not obtained for genomic species 13TU, since only one reference strain from a public culture collection was included. The results demonstrated the high degree of conservation of both the ITS length and the ITS sequence in (genomic) species of the *A. calcoaceticus-A. baumannii* complex. Table 2 shows the results of pairwise comparison of the ITS sequences between any two (genomic) species of *Acinetobacter*. In addition to differences in sizes, the sequences of multiple ITS fragments produced by a species also differed, as observed by cloning and sequencing of four ITSs (578, 614, 649, and 716 bp) from the type strain of *A. haemolyticus* (BCRC 14852) (Fig. 1, lane 4); the sequence similarities between any two of the four fragments varied from 0.75 to 0.94 (data not shown). Two ITS fragments (703 and 716 bp) with a sequence similarity of 0.86 were cloned for the type strain of *A. johnsonii* (BCRC 14853) (Fig. 1, lane 7). To simplify the comparison, only the shortest ITS of a species was used for pairwise comparison between any two (genomic) species in Table 2. Overall, a high level of interspecies divergence of the ITS sequences was observed, ranging from 0.48 (*A. johnsonii* versus *A. ursingii*) to 0.92 (*A. baumannii* versus genomic species 13TU). Relatively high degrees of similarity (0.86 to 0.92) were observed between the (genomic) species of the *A. calcoaceticus-A. baumannii* complex (Table 2). For the remaining species, the interspecies ITS similarities were generally less than 0.84; however, exceptions were found between genomic species 10 and 11 (0.88), 14TU and 15BJ (0.90), 15BJ and 17, and 16 and "*A. venetianus*" (0.92). On the basis of the ITS intraspecies similarity levels, it seemed feasible to identify a strain to a (genomic) species of the *A. calcoaceticus-A. baumannii* complex by comparison of the ITS sequences of the test strain and the reference strains.

Identification of clinical isolates of the *A. calcoaceticus-A. baumannii* complex by ITS sequencing. To further explore the use of ITS sequencing for identification of (genomic) species of the *A. calcoaceticus-A. baumannii* complex, 18 isolates of *A. calcoaceticus* and 61 isolates of *A. baumannii*, as identified with the API 20 NE system, were analyzed. In total, 46 isolates were identified as *A. baumannii* by ITS sequence analysis, which was in agreement with the phenotypic identification obtained with the API 20 NE system. Nineteen isolates were identified as genomic species 3 by ITS analysis. Of these, 17 had been identified as *A. calcoaceticus* and 2 had been identified as *A. baumannii* with the API 20 NE system. Eleven isolates were identified as genomic species 13TU by ITS analysis, while they had all been identified as *A. baumannii* with the API 20 NE system. Three isolates could not be identified by ITS analysis. One of these (LUH 8943) had been identified as *A. calcoaceticus*, and the others (LUH 8944 and LUH 8946) had been identified as *A. baumannii* with the API 20 NE system (Table 3). LUH 8943 had an ITS sequence similarity of 0.91 to genomic species 15TU, LUH 8944 had similarities of 0.93 to genomic species 3 and 0.91 to genomic species 13TU (BCRC 15417), whereas LUH 8946 had a similarity of 0.96 to both *A. baumannii* and genomic species 13TU (Table 3).

Two strains of each (genomic) species, as identified by ITS sequencing but for which the results of ITS sequencing analysis and identification with the API 20 NE system were discrepant, and strains that could not be identified by ITS analysis (strains LUH 8943, LUH 8944, and LUH 8946) were further investigated by both ARDRA and AFLP (Table 3). The results of these methods were in concordance for strains identified as either genomic species 3 or genomic species 13TU by ITS, with similarities of 0.99 to 1.00. Of the unidentified strains, LUH 8943, which had an ITS sequence similarity of 0.91 with the genomic species 15TU reference strain, could not be identified by ARDRA, as its ARDRA profile, 65113, is not in the ARDRA database (although the closest match in the ARDRA database was profile 62113, which is the profile for genomic species 15TU). By AFLP analysis, LUH 8943 was linked by $\geq$50% with other reference strains of genomic species 15TU, which identified the strain as this genomic species. In addition, the 16S rRNA gene sequence of LUH 8943 had a 99% similarity to that of genomic species 15TU CCUG 26390 (data not shown). LUH 8944 was identified by both ARDRA and AFLP as the unnamed genomic species close to 13TU (20), the sequence of which is not yet included in the ITS database (Table 3). Finally, LUH 8946 was identified as *A. baumannii* by both ARDRA and AFLP. In summary, 76 of the 79 clinical isolates were identified by ITS analysis, corresponding to an identification rate of 96.2%.

DISCUSSION

This study describes a new method for the identification of species within the *A. calcoaceticus-A. baumannii* complex. The method consists of amplification and sequencing of the ITS region, followed by comparison of this ITS sequence with those of the type and reference strains of *Acinetobacter* species. The procedure is similar to the one recently published for the identification of clinical isolates of viridans group streptococci (10). The whole procedure can be finished within 24 h, starting from the time of colony isolation. With an identification rate of 96.2%, the present method provides an accurate alternative for the identification of strains to the (genomic) species of the *A. calcoaceticus-A. baumannii* complex, which are difficult to differentiate by phenotypic characteristics. Furthermore, the

TABLE 2. ITS sequence similarity among 21 genomic species of *Acinetobacter*

Species and strain[a]	ITS length (bp)	Sequence similarity with the following species:																			
		A. calcoaceticus	*A. baumannii*	Genomic species 3	Genomic species 13TU	*A. haemolyticus*	*A. junii*	Genomic species 6	*A. johnsonii*	*A. lwoffi*	Genomic species 10	Genomic species 11	*A. radioresistens*	Genomic species 14TU	Genomic species 15TU	Genomic species 14BJ	Genomic species 15BJ	Genomic species 16	Genomic species 17	"*A. venetianus*"	*A. ursingii*
A. calcoaceticus LMG 1046^T	638																				
A. baumannii BCRC 10591^T	607	0.86																			
Genomic species 3 LMG 1035	619	0.89	0.91																		
Genomic species 13TU BCRC 15417	615	0.91	0.92	0.89																	
A. haemolyticus BCRC 14852^T	614	0.67	0.66	0.67	0.63																
A. junii BCRC 14854^T	706	0.62	0.61	0.65	0.63	0.61															
Genomic species 6 BCRC 15421	606	0.68	0.76	0.68	0.74	0.68	0.63														
A. johnsonii BCRC 14853^T	703	0.60	0.60	0.61	0.61	0.54	0.68	0.63													
A. lwoffii BCRC 14855^T	629	0.65	0.69	0.65	0.68	0.57	0.57	0.69	0.67												
Genomic species 10 BCRC 15423	613	0.71	0.68	0.71	0.69	0.67	0.65	0.69	0.65	0.65											
Genomic species 11 BCRC 15424	593	0.72	0.68	0.72	0.69	0.65	0.65	0.67	0.64	0.69	0.88										
A. radioresistens BCRC 15425^T	632	0.71	0.78	0.71	0.77	0.65	0.67	0.78	0.66	0.68	0.73	0.70									
Genomic species 14TU LMG 1235	667	0.67	0.69	0.72	0.69	0.71	0.76	0.76	0.67	0.63	0.71	0.70	0.74								
Genomic species 15TU CCUG 26390	661	0.61	0.65	0.62	0.65	0.59	0.65	0.67	0.70	0.70	0.66	0.63	0.73	0.66							
Genomic species 14BJ CCUG 34435	620	0.74	0.71	0.73	0.71	0.73	0.68	0.71	0.58	0.64	0.72	0.75	0.70	0.75	0.61						
Genomic species 15BJ CCUG 34436	667	0.67	0.67	0.71	0.67	0.67	0.77	0.77	0.67	0.63	0.73	0.71	0.72	0.90	0.69	0.74					
Genomic species 16 BCRC 15883	595	0.71	0.71	0.77	0.70	0.77	0.74	0.71	0.60	0.64	0.71	0.73	0.70	0.81	0.62	0.84	0.81				
Genomic species 17 CCUG 34437	655	0.69	0.70	0.73	0.70	0.67	0.79	0.74	0.67	0.62	0.76	0.72	0.74	0.84	0.69	0.75	0.92	0.81			
"*A. venetianus*" CCUG 45561	612	0.68	0.69	0.74	0.67	0.73	0.78	0.67	0.60	0.64	0.68	0.72	0.67	0.74	0.62	0.81	0.73	0.92	0.73		
A. ursingii LMG 19575	715	0.69	0.73	0.67	0.75	0.61	0.52	0.70	0.48	0.61	0.64	0.65	0.68	0.59	0.59	0.66	0.58	0.68	0.61	0.67	
A. schindleri LMG 19576	638	0.65	0.63	0.63	0.62	0.69	0.61	0.62	0.63	0.61	0.70	0.67	0.65	0.64	0.76	0.65	0.66	0.65	0.68	0.61	0.57

[a] See footnote *a* of Table 1 for the definitions of BCRC, LMG, CCUG, and ATCC.

TABLE 3. Clinical isolates of *Acinetobacter* that produced discrepant identifications by phenotypic tests (API 20 NE system) and molecular methods (ITS sequence analysis, ARDRA, and AFLP)

Isolate	Genomic species or species identified by:			
	API 20 NE	ITS sequence similarity[a]	ADRDA (profile no.)[b]	AFLP
LUH 8952	*A. calcoaceticus*	3 (1.00)	3 (21313)	3
LUH 8953	*A. calcoaceticus*	3 (1.00)	3 (21313)	3
LUH 8943	*A. calcoaceticus*	15TU (0.91)	NI[c]	15TU
LUH 8951	*A. baumannii*	3 (0.99)	3 (21313)	3
LUH 8950	*A. baumannii*	3 (0.99)	3 (21313)	3
LUH 8948	*A. baumannii*	13TU (1.00)	13TU (21111)	13TU
LUH 8949	*A. baumannii*	13TU (0.99)	13TU (21111)	13TU
LUH 8944	*A. baumannii*	3 (0.93), 13TU (0.91)	Close to 13TU	Close to 13TU
LUH 8946	*A. baumannii*	*A. baumannii* (0.96) 13TU (0.96)	*A. baumannii*	*A. baumannii*

[a] The type strain or the first reference strain listed in Table 1 was used as the basis for calculation of similarity.
[b] Combination of restriction patterns of the amplified 16S rRNA gene obtained by using CfoI, AluI, MboI, RsaI, and MspI. Identification is achieved by comparing the profile to those in a database of profiles (14, 44; http://allserv.rug.ac.be/~mvaneech/ARDRA/Acinetobacter.html).
[c] NI, not identified.

method has the potential to identify other named and unnamed *Acinetobacter* species since the primers used in this study (primers 1512F and 6R) could amplify the ITS regions of all 21 (genomic) species of *Acinetobacter* tested. However, the applicability of ITS sequencing for the identification of acinetobacters other than those in the *A. calcoaceticus-A. baumannii* complex needs to be validated by testing more reference strains and clinical isolates.

Taxonomically, the genus *Acinetobacter* has a rather long and complicated history (27, 34) and currently comprises up to 33 (genomic) species, including seven recently described environmental species (9) and the clinically relevant species *A. parvus* (35). The biology and clinical significance of different *Acinetobacter* species need to be established. Unfortunately, species identification is hampered by the lack of reliable yet practical methods. Phenotypic schemes have been worked out for the identification of *Acinetobacter* (7, 21, 29); but these methods require specific media, and several days of incubation are normally required. In addition, these methods have been shown to be problematic in some cases, especially for the separation of (genomic) species of the *A. calcoaceticus-A. baumannii* complex. Commercial identification systems, such as the API 20 NE system (4) and the VITEK 2 system (33), were also found to be inaccurate for the identification of strains as species of the *A. calcoaceticus-A. baumannii* complex and glucose nonfermenters, respectively.

Among the molecular methods developed for the identification of *Acinetobacter*, ARDRA, which is based on restriction analysis of amplified the 16S rRNA gene, and whole-genome high-resolution fingerprinting by AFLP (28) have been validated with a large set of reference strains of all described species (14, 34, 44), including those of the *A. calcoaceticus-A. baumannii* complex. Ribotyping also has a high discriminatory power for species identification and strain typing of members of the *A. calcoaceticus-A. baumannii* complex (19). However, the banding patterns of AFLP and ribotyping are very complex, and libraries of profiles of well-defined strains are required for species identification.

The 16S rRNA gene sequences of members of the genus *Acinetobacter* have an overall sequence similarity of more than 94%; and on the basis of analysis of their 16S rRNA gene sequences, strains of 21 (genomic) species of *Acinetobacter* were allocated to five clusters (27). *A. baumannii* and genomic species 13TU were in cluster 1, whereas *A. calcoaceticus* and genomic species 3 were in clusters 5 and 2, respectively. Unique motifs in each of the 21 DNA groups were distinguished, and direct sequencing of the 16S rRNA gene was proposed for the identification of *Acinetobacter* species (27). The usefulness of the 16S rRNA gene as a taxonomic marker was corroborated by restriction analysis of the 16S rRNA gene by ARDRA. However, it was learned that multiple ARDRA profiles may occur among different strains of the same species (suggesting intraspecies diversity), and therefore, an extensive library displaying both intra- and interspecies variations is required for species identification (14). Instead of the complete 16S rRNA gene sequence, the first 527 bp was reported to be sufficient for identification of the genus or species of unusual aerobic pathogenic gram-negative bacilli (42). However, because only three strains of *Acinetobacter* were included in that study, the question of the applicability of the first 527 bp of the 16S rRNA gene for the identification of members of the *A. calcoaceticus-A. baumannii* complex remains to be answered.

The ITS region has been suggested to be a good candidate for bacterial species identification (2, 10, 16, 22, 23). Pairwise comparison of any two species in the *A. calcoaceticus-A. baumannii* complex revealed a lower level of ITS sequence similarity (Table 2) than 16S rRNA gene similarity (27), indicating that the ITS region constitutes a more discriminative target sequence than the 16S rRNA gene. Compared to the 16S rRNA gene (≈1.5 kb), the ITS fragments in the *A. calcoaceticus-A. baumannii* complex are relatively short (607 to 638 bp; Table 1), which makes sequencing of the ITS fragments easier and more straightforward.

In the present study, each of the four (genomic) species in the *A. calcoaceticus-A. baumannii* complex produced a single PCR product that facilitated direct sequencing (Fig. 1). However, multiple PCR products were produced by *A. haemolyticus*; *A. johnsonii*; *A. lwoffii*; and the unnamed genomic species 10, 11, 14TU, and 14BJ (Fig. 1), as has been reported for many bacterial species (12, 24). In these cases, direct sequencing of the PCR-amplified products is impossible, and sequencing should be done by cloning multiple ITS fragments. Therefore,

it seems that identification of (genomic) species with multiple ITS fragments would be more difficult than identification of species of the *A. calcoaceticus-A. baumannii* complex. In this context it is noteworthy that Nowak et al. (38) found that species of the *A. calcoaceticus-A. baumannii* complex produced several different ITS fragments by PCR amplification, but Dolzani et al. (16) found only one PCR product in strains of the *A. calcoaceticus-A. baumannii* complex, which is in agreement with our findings.

The clinical isolates used in this study had been identified as either *A. calcoaceticus* or *A. baumannii* with the API 20 NE system. It was not the purpose of the present study to evaluate this commercial system, since its poor performance for the identification of species of the *A. calcoaceticus-A. baumannii* complex has already been documented (4). Nevertheless, the present results reemphasize the inaccuracy of the system. In this study, 17 of 18 clinical isolates identified as *A. calcoaceticus* with the API 20 NE system were actually genomic species 3, as identified by ITS sequencing; and their identities were further confirmed by ARDRA and AFLP. Therefore, the API 20 NE kit is considered unacceptable for species identification, since *A. calcoaceticus* is an environmental species without clinical significance (6).

In conclusion, identification of species of the *A. calcoaceticus-A. baumannii* complex by ITS sequencing is reliable and straightforward if a DNA sequencer is available in the clinical setting. Alternatively, if DNA sequencing facilities are not available, the ARDRA method, which has been proved to be useful for the identification of *Acinetobacter* species (14, 36), may be an alternative choice, since a PCR machine and a gel electrophoresis apparatus are normally accessible. The present approach may help to elucidate the ecology and clinical significance of the different species in this complex. However, the database should first be extended with well-validated strains to inventory the variations within species.

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